Biochimica et Biophysica Acta, 603 (1980) 347—365

BBA 79038

MODIFICATIONS OF MEMBRANE LIPID COMPOSITION FOLLOWING THE NUTRITIONAL SHIFT-UP OF STARVED CELLS

A COMPARISON WITH MEMBRANE BIOGENESIS IN *TETRAHYMENA*

YOSHINORI NOZAWA, REIKO KASAI and TAKASHI SEKIYA

Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu (Japan)

(Received June 16th, 1980)

Key words: Lipid composition; Membrane biogenesis; Starvation; Phospholipid; Ameliorative modification; (Tetrahymena)

Summary

Detailed analyses of lipid composition have been made on various membrane fractions isolated at different intervals after 24 h-starved *Tetrahymena* cells were refed with nutrient-rich medium. During starvation there was a marked alteration in both phospholipid polar headgroup and acyl chain compositions: an increase in 2-aminoethylphospholipid and γ-linolenic acid (18 : 3) with a concurrent decrease in phosphatidylethanolamine and palmitoleic acid (16 : 1). However, following refeeding, such an altered lipid composition was rather rapidly restored to the initial level of the control cell membranes prior to starvation. This membrane lipid modification was found to occur in good accordance with the recovery of cell size and lipid synthesis. The considerable changes in the principal unsaturated fatty acids, 16 : 1 and 18 : 3, which are formed via the palmitate and stearate desaturation pathways, respectively, were suggested to be accounted for by the levels of desaturases activities. The results of the labeling experiments with radioactive precursors have demonstrated that in the refed cells, there was a more rapid and dynamic transfer or exchange between membranes as compared with that in the exponentially growing control cells. Thus, rapid ameliorative modifications of membrane lipid composition are thought to be required for the urgent growth of membrane systems in the refed cell which should be ready to initiate new division.

Introduction

It is widely documented that a variety of cells undergo modifications of membrane lipid composition in response to changes in growth conditions

such as temperature, nutrient, senesence, etc. Adjustment of lipid composition is thought to be required for maintaining normal functions taking place in membranes when cells are exposed to new, either comfortable or uncomfortable, environments. The most plausible explanation for such adaptive lipid modification is derived from the well accepted concept of the fluid mosaic membrane structure proposed by Singer and Nicolson [1]. Various membrane functions have been observed to be closely associated with membrane lipids via the lipid microenvironment and bulk membrane fluidity [2,3].

A unicellular eukaryote, *Tetrahymena*, which possesses most of the specialized membrane systems found in higher eukaryotic cells, has been shown to modify remarkably its membrane lipid composition depending upon culture conditions [4,5]. In addition, since its growth and division can be readily modulated by nutritional control, this organism may be a suitable model system for investigating the cell growth-associated lipid modification of membranes. As an initial attempt, we have examined alterations in membrane lipid composition occurring under two extreme nutritional conditions: shift-down (starvation) and shift-up (refeeding). In particular, more detailed analyses including radioisotopic labeling have been made on the membrane lipid changes following the refeeding of starved cells because the refed cells exert rapid and prominent modification of their membrane lipid profile, and therefore would yield certain useful information regarding the intracellular lipid mobilization associated with membrane renewal after nutritional shift-up.

The results obtained from this study indicated that the composition of the phospholipid polar hear groups as well as the acyl chains, was rapidly altered in various membranes of the refed cells, thus being restored within a short term to the initial level observed in the exponentially growing control cells prior to starvation. Some observations from radioactive-labeling experiments will also be discussed in terms of membrane assembly induced by nutritional shift-up.

Materials and Methods

Cell growth. Tetrahymena pyriformis NT-I, a thermotolerant strain originally isolated from a hot spring in New Mexico, was cultured axenically at 34°C in an enriched proteose peptone (Difco) medium as previously described [6]. The stocks were subcultured once a week. The cell number was counted with a hemocytometer.

Starvation (shift-down) and refeeding (shift-up). For starvation of cells, cells in the mid-logarithmic phase were harvested at $145 \times g$ for 5 min in sterile capped bottles in a Sorvall RC2-B centrifuge, and then resuspended to a final cell density of approx. $3-4 \cdot 10^5$ cells/ml in a non-nutrient medium (0.68 g K_2HPO_4, 0.15 g KH_2PO_4, 2.75 g NaCl, 0.25 g $MgSO_4 \cdot 7H_2O$, dissolved in 1 l of distilled water, pH 6.5) [7] and incubated at 34°C with gentle shaking. Care was taken not to damage the cells during preparation for starvation. At different intervals during starvation, cells were centrifuged at $164 \times g$ for 5 min for lipid analysis.

Conversely, for nutritional shift-up (refeeding), the cultures starved for 24 h were filtered through sterilized gauzes and then centrifuged at $164 \times g$ for

5 min. The harvested cells were resuspended in the same enriched nutrient medium as that used for cell growth prior to starvation.

Isolation of subcellular membrane fractions. The preparation of several different membrane fractions was performed essentially according to the method of Nozawa and Thompson [8,9]. Cells were harvested at different intervals during starvation and washed in a cold potassium phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/2 mM EDTA/0.1 M NaCl, pH 7.2). Cilia were detached from the cell bodies by gentle hand homogenization until most of cells ruptured leaving the pellicle ghosts intact, which were spun down at 1020 × *g* for 5 min and then purified by discontinuous density gradient centrifugation in sucrose (1.0 and 1.72 M). Mitochondria and microsomes were prepared from the particulate fraction by differential centrifugation at 19 600 × *g* for 20 min and at 105 000 × *g* for 60 min, respectively. Since the starved cells were smaller in size than the actively growing cells, some adjustments were required for determining the suspension volume and the number of strokes needed to disrupt cells.

Lipid extraction and analysis. Total lipids were extracted from isolated membrane fractions according to the method of Bligh and Dyer [10] with $CHCl_3/CH_3OH$. Phospholipid phosphorus content was determined according to the method of Bartlett [11] using 70% $HClO_4$ for decomposition. Individual phospholipids were separated on silica gel H thin-layer plate with a solvent system of $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (75 : 25 : 5 : 2.2, v/v). After charring the developed plate with 50% H_2SO_4, the areas corresponding to individual phospholipids were scraped off the plate and their phosphorus contents were determined by using the method of Rouser et al. [12]. Quantitative analysis of fatty acids was carried out by using gas-liquid chromatography as previously described [13]. Total lipids extracted from membrane fractions and individual phospholipids eluted from silica gels with $CHCl_3/CH_3OH$ (1 : 1, v/v) followed by $CHCl_3/CH_3OH$ (1 : 9, v/v) were methylated with BF_3/CH_3OH and the methyl esters were analyzed in a JEOL Model JGC-1100 gas chromatograph. The unsaturation index of fatty acid composition is defined as Σ[(number of double bonds in each fatty acid) × (mol% of each fatty acid)] [14].

Radioisotopic labeling. To determine the rate of lipid synthesis, aliquots (20 ml) of cell culture were periodically taken during starvation and refeeding, and labeled with 5 μCi of sodium [1-^{14}C]acetate (New England Nuclear, 58.3 mCi/mmol) for 30 min prior to lipid extraction. Also, the control and refed cells labeled with 0.5 μCi of [1-^{14}C] palmitic acid (New England Nuclear, 50.2 mCi/mmol) for desired periods of time, and several membrane fractions were isolated from the radioisotope-labeled cells as described above. The incorporation of these radioactive precursors into individual lipid fractions was measured by counting the radioactivity in areas corresponding to the lipid fractions separated on silica gel H plate. The assay of radioactivity was performed with a Beckman LS-9000 scintillation spectrometer. In order to investigate the distribution of the radioactivity in individual fatty acids, radioactive methyl esters separated by gas-liquid chromatography were trapped into glass tubes (5 mm × 15 cm) with two open ends and eluted from the tubes with toluene into glass vials for radioactivity assay [15].

Assay of palmitoyl-CoA and stearoyl-CoA desaturase activities. The standard

reaction mixture (1 ml) consisted of 40 μM [1-^{14}C]palmitoyl-CoA or [1-^{14}C]-stearoyl-CoA (1 mCi/mmol)/0.1 mM NADH/0.5—0.7 mg microsomal protein in 0.1 M potassium phosphate buffer, pH 7.2. After preincubation for 1 min at 34°C, the reaction was initiated by addition of the enzyme solution and terminated after 3-min incubation with continuous shaking. The analyses of reaction products were carried out according to the procedures described previously [16]. The specific activities of the desaturases were calculated as nmol palmitoleate or oleate formed/min per mg microsomal protein.

Protein content was assayed according to the method of Lowry et al. [17] using bovine serum albumin as standard.

Electron microscopy. Changes in size and surface features of cells during nutritional shift-down and shift-up were observed using a scanning electron microscope (JSM-U3) as previously described [18]. Cells were fixed for 1 h with a mixture of glutaraldehyde and 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4), then dehydrated in an acetone series, critical-point dried, and shadowed with carbon-gold. Ultrastructural observations of intracellular organelles in the starved and refed cells were performed using thin-sectioning electron microscopy. Samples were fixed for 50 min in 1% glutaraldehyde/OsO_4 in 0.1 M phosphate buffer at pH 7.1, and rinsed in the same buffer. After dehydration in a graded series of ethanol, the cells were embedded in styrene. Sections were cut on a Porter MT-2B ultramicrotome, stained for 15 min with 1% uranyl acetate followed by lead citrate, and examined in a JEM 100-U electron microscope.

Results

Ultrastructural changes of T. pyriformis NT-I during starvation and its consequent refeeding

After the exponentially growng cells of *T. pyriformis* NT-I were transferred into the non-nutrient P_i medium, some aliquots were taken at various intervals for observation of size and surface features by using scanning electron microscopy. Shortly after nutritional shift-down, cells undergo a gradual reduction in both length and diameter to approximately half the size of the initial control cells before starvation. However, the total number of cilia covering the whole body was unchanged even in the 24 h-starved cell. The apparent increase in the cilia density is due to the reduced cell surface area. The cell motility also was not different from that of control growing cells. Upon shift-up of 24 h-starved cells to the nutrient medium enriched with proteose/peptone/yeast extract, cells promptly started to regain their volumes and even 1 h after the shift a significant increase in cell diameter was observed. With the passage of time, a gradual but rather rapid recovery of cell size occurred, and the 24 h-refed cells became almost the same as the control cells. The overall profile of size changes during starvation and refeeding is summarized in Fig. 1. It should be noted that a few cells with some indication of division were first observed 6 h after refeeding of the 24 h-starved cells.

The ultrastructure of intracellular membrane systems was studied on thin sections after various periods of starvation and refeeding. Some representative features are shown in Fig. 2. The most obvious change occurring in the early

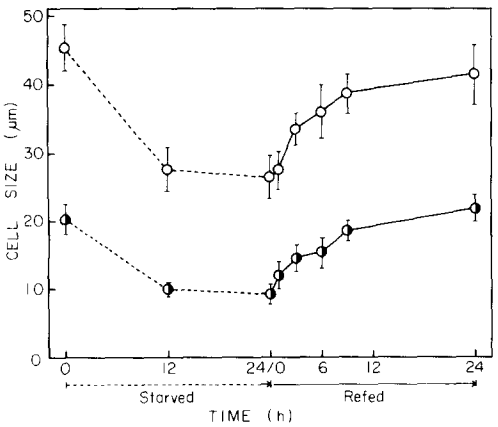

Fig. 1. A profile of changes in cell size during starvation and refeeding of *T. pyriformis* NT-I. Each point represents the mean from at least 20 cells and vertical bars denote the range of variations. Note the more rapid recovery of width than length at the early stages after refeeding. ○, length; ◑, width.

stages of starvation is the profound increase in degenerating mitochondria. These become round in shape and are sequestered into the autophagic vacuoles to be digested. In the later stages, the number of mitochondria and endoplasmic reticula was greatly decreased. Endoplasmic reticula were present only sparsely in the 24 h-starved cells. In contrast to the reduced amounts of certain organelles due to sequestration and subsequent digestion, there was a marked increase in the number of mucocysts surrounded by their own unit membrane, the function of which is still unknown. Many mucocysts move towards the cell surface and rest near the alveolar membranes until discharge occurs. The possible role of the mucocyst will be discussed later in terms of membrane biogenesis. We have also examined membrane structures by freeze-fracture electron microscopy, but no specific alteration was found during starvation. The density of membrane-intercalated particles was unchanged from the control growing cells; 2300—2500 particles per μm^2 in the protoplasmic faces of the plasma and nuclear membranes.

On the other hand, after nutritional shift-up of the 24 h-starved cells, a number of structural changes occur in the cell, as demonstrated in thin-sectioned samples (Fig. 2). The compactness of cytoplasmic components appears to be dependent on the density of glycogen and ribosomes. The endoplasmic reticulum is still not well developed, and is scarcely observed even 3 h after incubation in the enriched medium. The 9 h-refed cells which regain active division contain numerous mitochondria with oval or elongated shapes and typical endoplasmic reticula attached with ribosomes. Small numbers of mucocysts are observed, but most, if not all, rest in the interior of cells as immature forms. Similar ultrastructural features were also observed in the cells after 6 h of refeeding when some of cells are ready to divide.

Modification of lipid composition of whole cells during starvation in the inorganic medium

Almost immediately after transfer of the exponentially growing cells to the

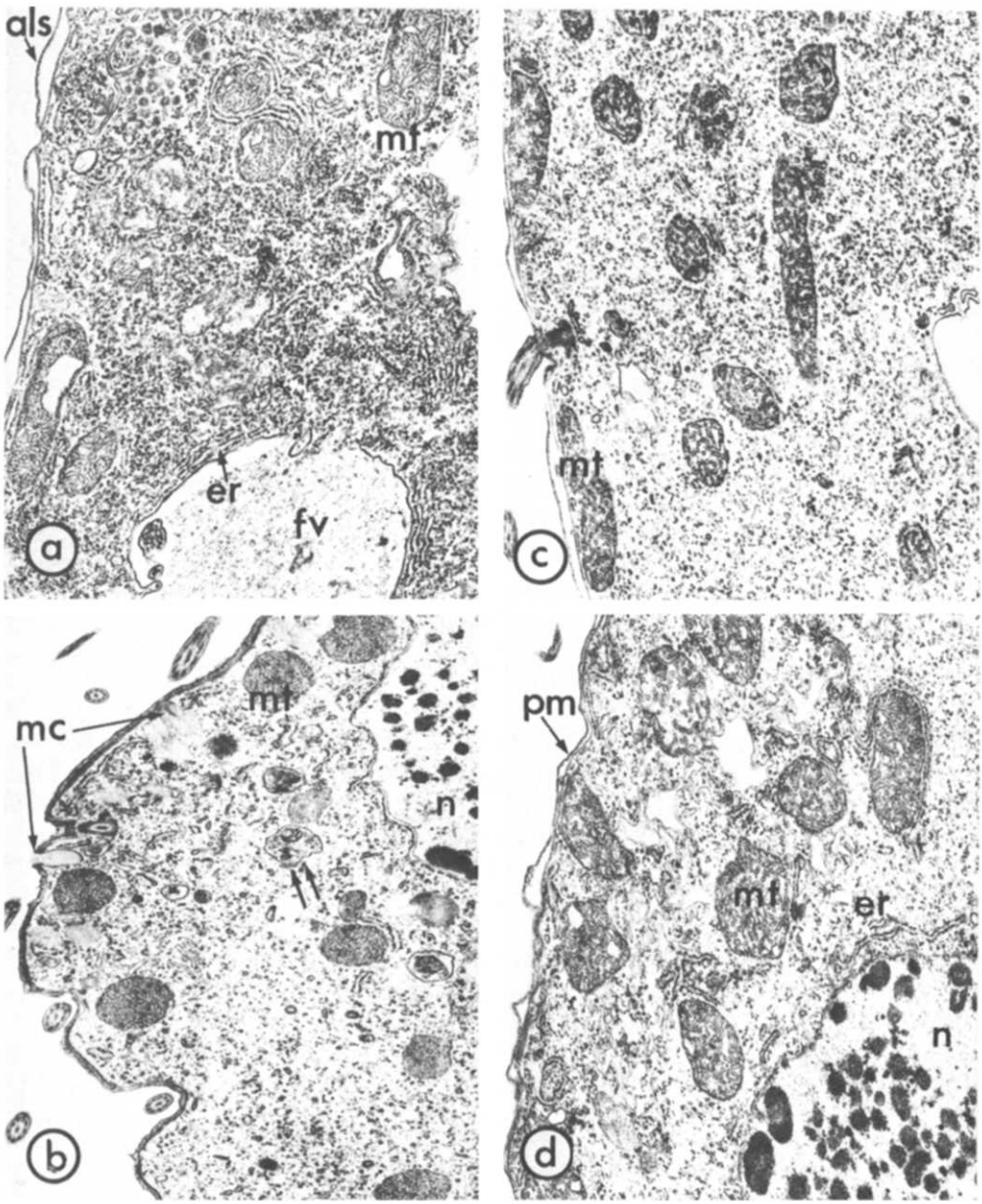

Fig. 2. Representative thin-section electron micrographs of *T. pyriformis* NT-I cells under starvation and refeeding conditions. (a) Control cell with well developed endoplasmic reticulum (er) and much glycogen; (b) 24 h-starved cell showing electron-dense mitochondria sequestered in the autophagic vacuoles (arrows). Note numerous mucocysts (mc) beneath the pellicular membrane to which they fuse for discharging; (c) 3 h-refed cell with some oval mitochondria (mt) having normal matrix space but still few endoplasmic reticula; (d) 9 h-refed cell with normal mitochondria and some developing endoplasmic reticula; (pm) plasma membrane; (als) alveolar sac; (fv) food vacuole; (n) nucleus. Magnification, × 14 000.

nutrient-deficient medium, a pronounced decrease in the content of phospholipids occurs, especially within the first 12 h period. Also, marked reduction of cell size was observed during this time (Fig. 1). The 24 h starvation decreases the phospholipid content in the cell by less than half relative to the control. Such a drastic decline of phospholipid content results not only from active autophagic digestion of organelles but also from the absence of lipid synthesis as inferred by much lowered incorporation of [^{14}C]acetate into lipid fractions. The relative specific activity (cpm/phospholipid) of the 24 h-starved cells was only a few percent of the control growing cells.

During the starvation period, there were marked changes in the composition of phospholipid polar head groups as well as acyl chains. The contents of phosphatidylethanolamine decreases gradually with a corresponding increase in 2-aminoethylphosphonolipid, which is highly resistant to enzymatic degradation and present in abundance in *Tetrahymena* [8]. Conversely, the level of phosphatidylcholine and cardiolipin remains rather consistent. The fraction of phosphatidylserine plus phosphatidylinositol shows a slight increase. The fatty acyl chain composition also was observed to be modified following starvation. Especially during the first 4 h incubation in non-nutrient medium, various fatty acids undergo large changes. The proportion of the polyunsaturated fatty acids, oleic (18 : 1 [9]), linoleic (18 : 2 [9,12]) and γ-linolenic (18 : 3 [6,9,12]) acids, increases, whereas the level of palmitoleic (16 : 1 [9]), myristic (14 : 0) and palmitic (16 : 0) acids exhibits a concurrent decrease. These proportional alterations result in a much higher ratio of unsaturated to saturated fatty acid contents. At 4 h of starvation, linoleic acid begins to decline and reaches the initial level. However, γ-linolenic acid continues to increase at the expense of palmitoleic acid. Other fatty acids show little or no change. During the course of starvation, there is a continuous enhancement of the unsaturation index, defined as Σ[(number of double bonds in each fatty acid) $\times$ (mol% of each fatty acid)], which is largely dependent upon the increase in γ-linolenic acid. These results indicate that phospholipids of starved cells contain more double bonds per acyl group.

In order to determine which phospholipid(s) plays a major role in modifying the phospholipid acyl group profile in the starved cells, gas chromatographic analysis was performed on three major phospholipids. The results are summarized in Table I, demonstrating that the general trend observed in total phospholipids, an increase in γ-linolenic acid with a concurrent decrease in palmitoleic acid, is reflected to varying degrees in all phospholipids. The smallest changes are found to occur in 2-aminoethylphosphonolipid, showing an 8% increase in 18 : 3 and a 5% decrease in 16 : 1. In contrast, the most striking alterations in the distribution pattern of acyl chains are seen with phosphatidylcholine in that 18 : 3 content increases gradually with time of starvation up to 50% at 24 h. It is of interest to note that the final composition is very similar to or even identical with that of 2-aminoethylphosphonolipid. Changes in the phosphatidylethanolamine acyl chain composition appear to be rather intermediate between those of phosphatidylcholine and 2-aminoehtylphosphonolipid, with 18 : 3 increasing by 15% and 16 : 1 decreasing by 12%. Considering the proportional changes in both fatty acyl chain and phospholipid class composition, it seems that phosphatidylcholine and 2-aminoethyl-

TABLE I

MODIFICATION DURING STARVATION OF ACYL CHAIN COMPOSITION OF WHOLE CELL PHOSPHOLIPIDS

The results are averages of two experiments and are expressed as relative percentages of total fatty acids. The error range was less than 3% of each value.

Fatty acids	Phosphatidylcholine					Phosphatidylethanolamine					2-Aminoethylphosphonolipid				
	Control	Starved for:				Control	Starved for:				Control	Starved for:			
		4 h	6 h	12 h	24 h		4 h	6 h	12 h	24 h		4 h	6 h	12 h	24 h
12 : 0	0.6	0.3	0.2	0.1	0.3	3.4	1.0	0.6	0.6	0.6	0.2	0.4	0.2	0.2	0.1
14 : 0	10.0	5.4	5.7	5.0	4.8	13.2	9.8	8.5	9.7	9.8	4.0	4.3	3.3	2.4	4.2
15 : 0	1.3	2.1	2.8	3.6	4.7	1.5	2.6	3.1	5.8	8.0	0.7	1.5	1.4	1.6	4.3
16 : 0	11.2	9.5	11.5	10.7	9.5	12.1	11.0	11.5	11.4	10.9	7.8	6.3	6.2	5.3	6.6
16 : 1	14.1	9.3	8.1	5.5	3.7	17.5	12.2	10.6	8.3	5.1	7.8	6.9	5.8	3.5	2.6
16 : 2 *	7.9	6.5	5.6	4.3	2.2	6.1	5.0	4.5	3.5	2.2	3.4	3.1	2.8	2.0	1.3
18 : 0	3.3	3.0	2.7	2.1	1.9	2.6	1.8	2.6	1.5	1.3	3.2	2.1	2.1	1.2	1.0
18 : 1	6.2	6.2	5.3	5.2	4.8	4.9	11.3	10.9	10.3	8.9	6.3	9.6	9.0	8.2	6.7
18 : 2 ***	3.2	4.1	4.4	5.2	6.3	1.3	1.1	1.4	1.9	2.3	6.8	7.9	8.8	10.4	10.9
18 : 2	12.0	13.1	12.2	12.2	9.4	12.1	17.7	18.2	18.6	16.1	12.5	13.6	13.4	11.3	10.0
18 : 3	24.1	36.6	36.6	42.1	49.9	14.4	19.6	20.3	23.0	29.4	41.4	42.5	45.4	51.6	49.2
Unsaturated (U)	59.6	69.3	66.6	70.2	74.1	50.2	61.9	61.4	62.1	61.8	74.8	80.5	82.4	85.0	79.4
Saturated (S)	26.4	20.3	22.9	21.5	21.2	32.8	26.2	26.3	29.0	30.6	15.9	14.6	13.2	10.7	16.2
U/S	2.26	3.41	2.91	3.27	3.50	1.53	2.36	2.33	2.14	2.02	4.70	5.51	6.24	7.94	4.90
Unsaturation index **	123.0	159.7	156.4	171.8	189.6	92.4	119.9	121.6	128.6	139.0	176.9	187.0	195.4	209.9	198.7

* Contains also small amounts of 17 : 0 which was not separable from 16 : 2 under the conditions used.

** Denotes the number of double bonds per each fatty acyl chain and was calculated from Σ[(number of double bonds in each fatty acid) $\times$ (percentage of the fatty acid)].

*** 18 : 2 (6, 11).

phosphonolipid may participate in the great increase of 18 : 3 and phosphatidylethanolamine and phosphatidylcholine in the marked decrease of 16 : 1. Another interesting finding is that pentadecanoic acid (15 : 0), a minor component in the control growing cell, gives rise to a gradual enhancement during starvation, being most remarkable in phosphatidylethanolamine.

Modifications of lipid composition in various membrane fractions after nutritional shift-up (refeeding)

Upon transferring the 24 h-starved cells into the proteose/peptone medium enriched with glucose and yeast extract, the cells started to ameliorate the markedly altered lipid content and composition which occurred under the starvation condition. Fig. 3 shows the changes in the phospholipid content of *T. pyriformis* NT-I after refeeding, indicating its rapid increase within the first 6 h period. This may result from the enhanced synthesis of phospholipids as indicated by the linear increase in the specific radioactivity obtained with [^{14}C]acetate. Thin-layer chromatographic analysis of the radioactivity distribution revealed that the radioactivity was incorporated almost entirely into the phospholipid fraction regardless of the period of refeeding. It is of importance to note that despite such a pronounced increase in the phospholipid content per cell, no indication of cell division was observed in the first 5.5 h incubation in the enriched medium. This indicates that the newly synthesized phospholipids are rapidly being utilized for renewal of intracellular

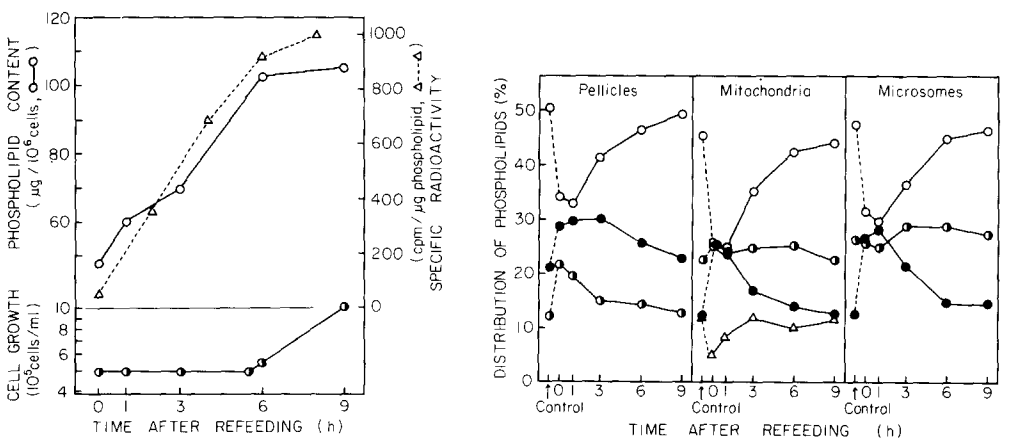

Fig. 3. Recovery of cell growth, phospholipid content, and lipid synthesis after refeeding of 24 h-starved cells of *T. pyriformis* NT-I. Cells taken at different intervals indicated following refeeding were labeled with [^{14}C]acetate for 30 min, and subjected to lipid extraction for measurements of the radioactivity and phosphorus content. Each point represents the mean from three separate experiments.

Fig. 4. Modification following refeeding of phospholipid polar head group composition of various membrane fractions of *T. pyriformis* NT-I cells. The 24 h-starved cells were transferred to proteose/peptone medium enriched with glucose and yeast extract, and various membrane fractions were isolated for lipid analysis. Each point represents the mean from three to four experiments except the control (six experiments). ○, phosphatidylethanolamine; ●, 2-aminoethylphosphonolipid; ◑, phosphatidylcholine; △, cardiolipin.

organelles which were lost during starvation by sequestration into autophagic vacuoles.

For determining which phospholipid components were responsible for the increased phospholipid content in the cell, the following membranes were chosen for further phospholipid fractionation: pellicles, mitochondria and microsomes. The results are shown in Fig. 4, which indicates a common but conspicuous trend towards an increase in phosphatidylethanolamine and towards a decrease in 2-aminoethylphosphonolipid, in contrast to that seen during the course of starvation. By way of this ameliorative modification, the levels of all major phospholipids recover to those of the control membranes prior to starvation. There are some good similarities in the phospholipid changes between two internal membranes, mitochondria and microsomes, i.e., an increase in phosphatidylethanolamine with the corresponding decrease in 2-aminoethylphosphonolipid, but little change in phosphatidylcholine. However, in the surface membrane pellicle there is an approx. 10% elevation in the percentage of phosphatidylcholine. A small but significant rise in cardiolipin level is also observed.

As for modification of the phospholipid fatty acyl chain composition after shift-up, similar or more profound changes observed in the whole cells are reflected in all isolated membrane fractions (Fig. 5). The most striking changes occur in 18 : 3 and 16 : 1, the former decreasing and the latter decreasing in nearly equal proportions. Linoleic acid (18 : 2) also shows a significant reduction during the initial 3 h refeeding period. There are small, if any, changes in other fatty acids. Thus, ameliorative alterations of the overall fatty acid compo-

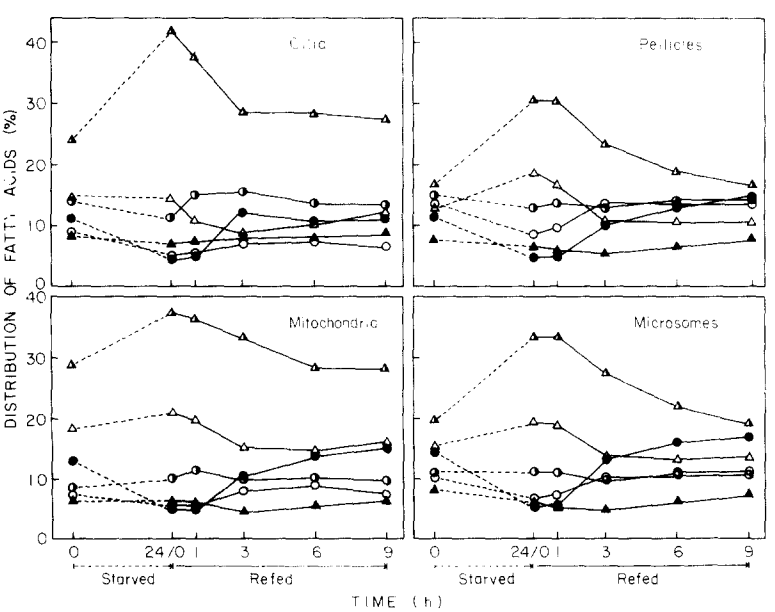

Fig. 5. Modification following refeeding of fatty acyl chain composition of various membrane fractions of *T. pyriformis* NT-I. Lipid extracted from various membrane fractions were analysed using gas-liquid chromatography. Each point represents the mean from three to four experiments. ▲, 18 : 3; △, 18 : 2; ●, 16 : 1, ◑, 16 : 0; ○, 14 : 0; ▲, 18 : 1.

sition appear to be almost completed by 6 h after the 24 h-starved cells were refed. It is demonstrated that, except for γ-linolenic acid undergoing a more than 10% decline within 3 h of refeeding, other fatty acids are modified least in the ciliary fraction in relative proportions. Such smaller alterations can probably be understood from the finding that the long period (24 h) of starvation did not affect the number and size of cilia as indicated by scanning electron-microscopic observations (Fig. 1), whereas other membrane fractions need active expansion or renewal for cell division.

It was possible to expect that, with respect to the extent to which the fatty acid composition was modified, more pronounced variations occurred among individual phospholipids extracted from different membrane fractions. Therefore, the fatty acyl chains of each phospholipid from pellicular, mitochondrial and microsomal membranes isolated at various intervals of refeeding were analysed. As indicated in Table II, the trend towards a decrease in γ-linolenic acid and towards an increase in palmitoleic acid holds true to different extents for all of the phospholipids. In these three membrane fractions, the decline of 18 : 3 content is most appreciable in phosphatidylcholine and 2-aminoethylphosphonolipid, whereas the enhancement of the 16 : 1 content is most conspicuous in phosphatidylethanolamine. For instance, in the case of mitochondria, 2-aminoethylphosphonolipid produces a 34% decrease in 18 : 3 combined with a 10% increase in 16 : 1, but phosphatidylethanolamine shows an 18% decrease and a 15% increase, respectively. In any case, it is noted that the specific phospholipid (2-aminoethylphosphonolipid) makes the greatest contribution towards decreasing the 18 : 3 content in all cell membrane fractions. Furthermore, this phospholipid contains a considerable amount of an unusual unsaturated fatty acid, cilienic acid (18 : 2 [6,11]) [19], which also shows a significant decrease during refeeding. Therefore, since 2-aminoethylphosphonolipid has the greatest number of double bonds per acyl chain, as inferred by the greatest unsaturation index (see Table II), the changes in its proportional content affect considerably the overall fatty acyl chain profile during the 9 h period of refeeding. Another finding to note is an appreciable rise in the 12 : 0 level, being restricted solely to phosphatidylethanolamine of the pellicular membrane and not to other phospholipids of this membrane nor even in the phosphatidylethanolamines of two other membrane fractions.

Activities of fatty acid desaturation during refeeding

As clearly demonstrated above, pronounced modifications induced during refeeding in the proportional composition of phospholipid fatty acyl chains can be largely accounted for by the increase in 16 : 1 and the decrease in 18 : 3. Therefore, it was expected that the activity of fatty acid desaturation might be altered in the refed cells. There exist two main pathways in *Tetrahymena* for the fatty acid unsaturation [20], the palmitate and stearate pathways. The former participates in the conversion of 16 : 0 to 16 : 1, whereas the latter is involved in the transformation of 18 : 0 to 18 : 3 via the sequence, 18 : 0 → 18 : 1 → 18 : 2 → 18 : 3. First, we examined the palmitoleate pathway. Cells taken at different intervals after nutritional shift-up were labeled with [^{14}C]palmitic acid and lipids were extracted for measurements of radioactivity in the 16 : 1 fraction separated by gas-liquid chromatography. The results are

TABLE II

MODIFICATION FOLLOWING NUTRITIONAL SHIFT-UP OF PHOSPHOLIPID ACYL CHAIN COMPOSITION IN THE PELLICULAR, MITOCHONDRIAL AND MICROSOMAL MEMBRANES

The results are averages of two experiments and are expressed as relative percentage of total acids, with the error range being less than 3% of each value: symbols as in Table I.

Phospho-lipid	Mem-brane	Fatty acyl chain composition (mol% of total)										Unsa-turated	Satu-rated	Unsatu-ration index
		12 : 0	14 : 0	15 : 0	16 : 0	16 : 1	16 : 2 *	18 : 1	18 : 2 **	18 : 2	18 : 3			
Phosphatidylcholine														
Refed 0 h	Pellicular	0.9	6.0	4.9	13.0	3.9	2.6	3.2	4.9	16.1	39.8	67.9	24.8	168.5
1 h		0.1	10.0	6.2	18.3	4.5	2.6	4.0	4.2	12.9	32.8	58.4	34.6	141.1
3 h		0.5	13.3	3.7	15.1	11.6	5.4	5.2	3.7	11.3	24.8	56.6	32.6	121.2
6 h		0.6	11.0	2.8	15.5	15.5	6.6	6.4	3.6	10.4	20.6	56.5	29.9	111.7
9 h		0.5	11.5	2.7	15.8	13.8	6.0	9.0	3.7	11.4	19.0	56.9	30.5	110.0
Control		0.5	11.6	2.0	16.7	12.8	7.0	10.8	2.0	11.8	16.6	54.0	30.8	101.0
Phosphatidylethanolamine														
Refed 0 h		0.2	8.8	7.4	15.2	4.5	2.1	7.7	1.5	24.2	21.2	59.1	31.6	127.2
1 h		1.7	14.5	7.7	17.0	5.4	2.3	8.0	1.6	17.7	18.0	50.7	40.9	106.0
3 h		5.4	18.0	5.4	15.7	9.6	3.6	7.1	1.5	11.8	13.2	43.2	45.5	82.9
6 h		5.4	16.6	3.7	15.5	16.3	5.9	7.0	1.4	10.6	9.7	45.0	41.2	76.4
9 h		7.3	15.4	3.0	15.8	15.3	5.2	9.0	0.6	10.8	8.5	44.2	41.5	72.6
Control		7.1	16.6	2.3	16.4	13.6	5.4	9.0	—	11.5	7.6	41.7	42.4	68.4
2-Aminoethylphosphonolipid														
Refed 0 h		—	0.6	0.9	3.8	1.2	0.9	4.1	11.6	11.4	63.8	92.1	5.3	242.7
1 h		0.3	6.7	3.8	9.7	3.6	1.9	6.7	8.9	13.0	42.1	74.3	20.5	180.4
3 h		0.6	7.9	2.5	9.5	5.9	2.5	7.1	8.5	10.3	41.4	73.2	20.5	174.8
6 h		0.5	6.4	1.7	8.0	10.0	3.8	7.1	10.0	10.3	37.5	74.9	16.6	170.2
9 h		0.5	6.1	1.2	7.3	11.4	4.7	8.2	9.5	12.1	35.1	76.3	15.1	168.1
Control		0.4	5.2	0.9	8.2	9.9	4.7	8.2	6.8	13.6	35.8	74.3	14.7	166.3
Phosphatidylcholine														
Refed 0 h	Mito-	—	2.2	2.4	11.1	3.1	2.5	2.9	6.1	16.1	49.8	78.0	15.7	199.8
1 h	chondrial	0.1	5.0	3.5	12.7	3.7	2.7	4.3	5.4	16.2	43.3	72.9	21.3	181.1
3 h		0.2	9.6	3.4	13.7	8.1	4.6	4.2	5.4	11.3	34.7	63.7	26.9	149.8
6 h		0.4	10.0	2.5	12.6	12.6	6.8	5.4	5.3	10.5	28.3	62.1	25.5	134.5
9 h		0.5	6.4	1.4	10.5	13.9	6.6	8.8	5.4	13.7	27.0	68.8	18.8	141.9
Control		0.4	7.8	1.4	11.1	11.6	7.5	9.6	3.5	12.6	26.7	64.0	20.7	133.5

Phosphatidylethanolamine													
Refed 0 h		—	3.1	3.2	7.4	3.4	1.7	5.0	1.5	30.3	41.2	81.4	13.7	195.6
1 h		0.4	8.1	4.5	9.5	5.4	2.6	6.3	2.0	23.6	32.9	70.2	22.5	161.6
3 h		1.4	10.2	2.8	9.1	11.5	3.8	5.7	1.8	16.5	30.5	66.0	23.5	145.3
6 h		2.1	9.9	1.8	8.8	19.7	6.8	5.8	1.8	12.0	24.1	63.4	22.6	125.4
9 h		1.7	7.7	1.3	8.5	18.7	5.6	7.7	1.7	15.6	25.5	69.2	19.2	137.5
Control		1.3	8.1	1.1	9.6	17.7	6.5	8.8	—	16.8	22.6	65.9	20.1	127.9
2-Aminoethylphosphonolipid														
Refed 0 h		—	0.3	0.2	1.8	0.8	0.6	2.8	13.0	9.8	69.2	95.6	2.3	256.8
1 h		—	3.1	2.0	6.0	2.7	1.5	5.6	11.7	12.5	53.2	85.7	11.1	216.3
3 h		—	4.9	1.7	7.9	6.5	2.3	6.8	10.5	11.4	45.1	80.3	14.5	192.4
6 h		0.4	5.5	1.3	7.0	11.7	4.2	6.6	10.4	11.2	37.6	77.5	14.2	174.3
9 h		0.4	4.5	1.0	6.7	11.7	4.6	8.2	9.8	14.4	35.2	79.3	12.6	173.9
Control		0.1	5.1	0.9	8.6	11.4	5.3	8.3	6.6	14.6	34.5	75.4	14.7	165.6
Phosphatidylcholine														
Refed 0 h	Micro-	—	4.0	3.5	9.8	3.5	2.9	3.0	5.8	16.3	46.5	75.1	17.3	190.2
1 h	somal	—	7.5	5.2	14.6	4.7	2.9	4.6	4.8	14.9	36.4	65.4	27.3	157.9
3 h		0.3	11.2	3.6	11.7	11.0	6.1	4.7	4.5	12.2	28.7	61.1	26.8	135.2
6 h		0.7	13.0	2.9	12.1	16.8	8.4	6.4	3.5	11.8	18.2	56.7	28.7	108.4
9 h		0.5	11.8	2.7	13.9	14.3	6.6	9.2	3.6	12.2	18.1	57.4	28.9	109.4
Control		0.4	10.4	1.9	12.9	13.1	7.8	10.8	2.3	12.9	18.9	58.0	25.6	111.0
Phosphatidylethanolamine														
Refed 0 h		0.1	8.3	6.4	11.4	4.7	2.5	5.2	1.9	26.3	27.5	65.6	26.2	148.8
1 h		0.6	11.6	6.1	12.3	6.2	2.7	6.6	2.2	21.6	24.8	61.4	30.6	134.8
3 h		1.8	12.4	3.4	9.7	13.1	5.8	5.5	2.1	15.7	24.1	60.5	27.3	126.5
6 h		2.8	12.8	2.2	10.0	21.7	7.7	6.2	1.7	10.8	15.4	55.8	27.8	99.1
9 h		1.8	10.9	2.0	11.1	20.1	6.7	8.8	1.7	14.2	14.9	59.7	25.8	105.4
Control		1.6	11.2	1.5	11.1	18.1	7.0	9.1	—	16.4	15.2	58.8	25.4	105.6
2-Aminoethylphosphonolipid														
Refed 0 h		—	1.0	1.4	4.4	1.7	1.4	3.7	10.5	14.1	60.8	90.8	6.8	237.0
1 h		0.1	4.4	2.5	7.2	3.7	1.8	5.6	10.3	13.1	49.0	81.7	14.2	203.1
3 h		0.3	5.1	1.7	6.7	6.6	2.7	6.0	10.5	11.8	45.6	80.5	13.8	194.0
6 h		0.5	6.6	1.5	6.6	13.3	5.3	6.2	9.1	11.0	34.7	74.3	15.2	163.8
9 h		0.5	6.2	1.2	7.5	13.3	5.2	8.2	8.4	13.7	31.1	74.7	15.4	159.0
Control		0.3	5.3	1.0	9.6	11.9	5.3	8.9	5.8	14.3	31.8	72.7	16.2	156.4

* Contains also small amounts of 17 : 0 which was not separable from 16 : 1 under the conditions used.
** 18 : 2 (6, 11).

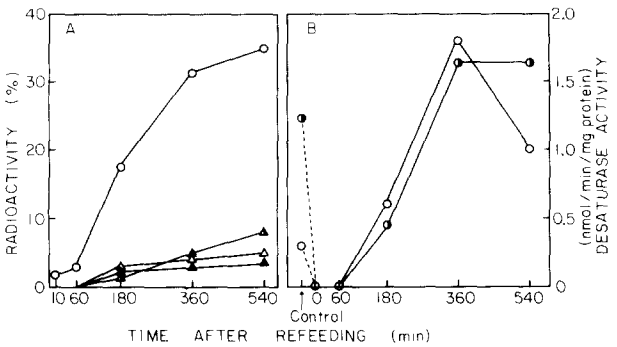

Fig. 6. Changes in desaturation activity following nutritional shift-up of the starved *T. pyriformis* NT-I cells. (A) Conversion rate of [^{14}C]palmitate to [^{14}C]palmitoleate in the refed cells. Immediately after the 24 h-starved cells were transferred into the nutrient-rich medium, they were labeled with [^{14}C]-palmitic acid, and thereafter aliquots were taken at the intervals indicated. The labeled lipids were examined for radioactivity in individual fatty acid methyl esters separated by gas-liquid chromatography. Each point represents the mean of two experiments. ○, 16 : 1; △, 18 : 2; ▲, 18 : 3; ▲, 18 : 1. (B) Activities of palmitoyl-CoA and stearoyl-CoA desaturases of microsomes. Microsomes were isolated at different intervals for measurements of palmitoyl-CoA and stearoyl-CoA desaturases as described in Materials and Methods. Each point represents the mean from two experiments with duplicate assays for each experiment. ○, palmitoyl-CoA desaturase; ◑, stearoyl-CoA desaturase.

illustrated in Fig. 6, which indicates a nearly linear enhancement by 6 h following shift-up in the conversion rate of [^{14}C]16 : 0 → [^{14}C]16 : 1, although within the first 1 h there is only very low desaturation activity. Such a markedly increased activity to form 16 : 1 leads one to predict the corresponding rise in palmitoyl-CoA desaturase activity involving the conversion of 16 : 0 to 16 : 1. These activities were eventually measured for microsomes isolated from cells at different periods of refeeding and were found to be well correlated with the results obtained in the in vivo labeling experiments with [^{14}C]-palmitate (Fig. 6). The maximal level of the activity observed at 6 h is almost 9-times as high as that of the control microsomes before starvation. At zero time, when the 24 h-starved cells were refed, there was little or no detectable activity of this enzyme. On the other hand, the much lowered proportion of γ-linolenic acid in the refed cells may be largely accounted for by the elevated 16 : 1 content and by the depressed activity of the stearate pathway in synthesizing γ-linolenic acid. An indication to suggest the latter possibility is the low radioactivity found in the 18 : 3 fraction as shown in Fig. 6, being less than half the radioactivity found in the control cells. However, the activity of stearoyl-CoA desaturase, catalyzing the transformation of 18 : 0 to 18 : 1, which involves the initial step in the desaturation sequence of the stearate pathway, first declined to an undetectable level after 24 h starvation but then started to rise to or even exceed the control level (Fig. 6). In addition, following refeeding, there was a gradual accumulation of radioactivity in the 18 : 0 and 18 : 1 fractions in [^{14}C]acetate-labeled cells (data not shown) which is reflected as an increase in the 18 : 1 content in phosphatidylcholine and 2-aminoethylphosphonolipid in all membrane fractions (Table II). Thus, these findings suggest that one or both steps after this initial desaturase process might be suppressed, but there is at the moment no available evidence for this assumption.

Intracellular mobilization of lipids during nutritional shift-up

As demonstrated earlier by ultrastructural observations, the reduction in cell size and the number of intracellular organelles (such as mitochondria and microsomes) per cell was found to occur after incubation in the nutrient-deficient medium, but upon refeeding the starved cells with enriched nutrient there was a rapid recovery of the membrane systems. It was also evident that such ameliorative membrane renewal was coupled with the modifications of lipid composition in various membrane fractions. Hence, a question was put forward as to how the newly made lipids are transferred or mobilized for expansion of the surface membrane, pellicle and renewal of some organelles. As an initial attempt to obtain information regarding this problem, we have pulse-labeled cells with [^{14}C]palmitate immediately after shift-up, and followed the distribution pattern of the radioactivity in various membrane fractions isolated at different periods of refeeding. Fig. 7 depicts the overall profile of radioactivity incorporation into the lipid fractions of several cell fractions from refed and control cells. In the control cells with exponential growth, 10 min after the addition of [^{14}C]palmitate, the highest specific activity is found in the microsomal fraction, with other fractions being much less labeled. The cilia and ciliary supernatant, which are metabolically isolated because of their remoteness from the sites of synthesis, show considerably lower specific activities. However, with the passage of time, the microsomal and supernatant fractions tend to decline in the level of specific activities, whereas the levels of other membrane fractions rise gradually. These fluctuations lead finally to the more or less similar level of specific activity at the 6 h-labeling time, suggesting a dynamic movement of lipids between different membranes. As

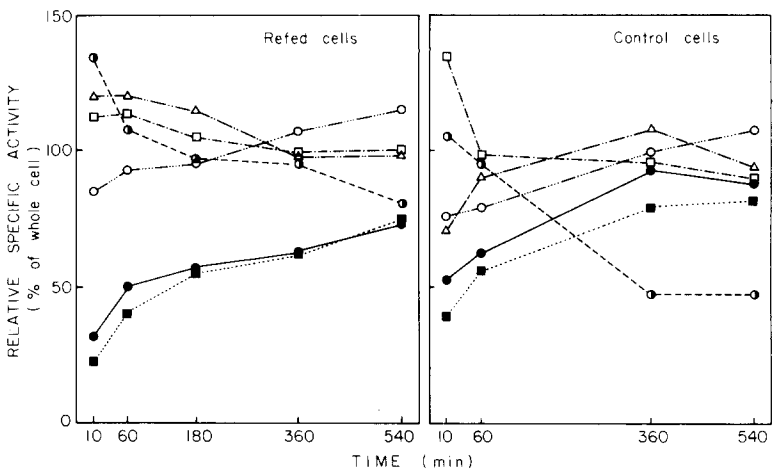

Fig. 7. Incorporation of [^{14}C]palmitic acid into total phospholipids of individual subcellular membrane fractions in the refed and control growing cells of *T. pyriformis* NT-I. After the radioisotope was added at zero min, cells were harvested at desired intervals for cell fractionation and lipids were extracted from the isolated membrane fractions for determination of radioactivity in the phospholipid fractions separated on thin-layer chromatography plate. Each point represents the mean from three to four experiments. Specific activity is given as cpm per μg phospholipid, and the relative specific activity is denoted as the relative percentage of the specific activity obtained for whole cells, △, pellicles; □, microsomes; ○, postmicrosomal supernatant; ○, mitochondria; ●, ciliary supernatant; ■. cilia.

opposed to the case of the control cells, the refed cells have smaller variations in specific activity among membrane fractions, except for the cilia and ciliary supernatant fractions. At the 10 min-labeling time, the microsomal fraction has already a much lower specific activity than the corresponding fraction from the control cells, thus being overwhelmed by the pellicular fraction. This might imply that the more rapid process of lipid transfer is occurring during the acute period for expansion of the surface membrane and pellicle and biogenesis of other organelles. The markedly low radioactivities in the cilia can be explained by the findings that its number and length remained unchanged during starvation; there is no need for the rapid supply of newly synthesized lipids.

Since over 80% of the total radioactivity is incorporated into phospholipids, the distribution of radioactivity was analyzed for three major phospholipids (data not shown). These labeling patterns offer an explanation for the modification in phospholipid class composition as seen in Fig. 4. Incubation in the enriched medium produced a substantial increase in the percentage of radioactivity in phosphatidylethanolamine in all cell membrane fractions.

Discussion

Numerous studies have provided substantial evidence that lipids exert a great influence on important biological activities occurring in membranes in the cell [32,34]. It is well known that in response to growth conditions, membrane lipid composition can be altered in a variety of microorganisms. However, by the recent extensive works in our and other laboratories, a unicellular eukaryote, *Tetrahymena*, has been proved to undergo a remarkable, either qualitative or quantitative, modification of membrane lipids under changed growth conditions [4,5,21]. These include (a) supplementation; sterols [18,22—25], fatty acids [13,26—28] and choline analogs (Kasai, R. and Nozawa, Y., unpublished results); (b) drugs [25,29,30]; (c) aging [31]; and (d) temperature [13,15,20,32—34]. In addition, we have demonstrated that coupled with the reduction in cell size, there was a marked decrease in the phospholipid-to-tetrahymanol ratio in starved *Tetrahymena* cells, but unexpectedly, a preliminary analysis using thin-layer chromatography displayed no large alterations in phospholipid distribution during starvation [35]. However, in the present study, more detailed examinations of lipid composition have been performed on cells during starvation, clearly indicating that pronounced modification not only in the phospholipid polar headgroup but also in the acyl chain composition. Upon transferring the exponentially growing control cells into the nutrient-deficient inorganic medium, a gradual decrease in phosphatidylethanolamine and a corresponding increase in 2-aminoethylphosphonolipid were found. As a plausible explanation for the mechanism by which this specific lipid-containing carbon-phosphorus bond is accumulated, its strong resistance against enzymatic degradation should be considered. Under conditions which do not permit net lipid synthesis, such stable lipids remain intact while other phospholipids such as phosphatidylethanolamine and phosphatidylcholine are decomposed by enzymatic attack. Therefore, it is possible that 2-aminoethylphosphonolipid could serve as an essential

lipid component in membranes for the survival of starved cells.

Furthermore, the starvation also induced a marked alteration in the fatty acyl chain composition. The general trend was an increase in the polyunsaturated fatty acids, oleic (18 : 1), linoleic (18 : 2) and γ-linolenic (18 : 3) acids, and a concurrent decrease in palmitoleic (16 : 1) acid. This suggests that in the two main pathways found in *Tetrahymena* to synthesize unsaturated fatty acids [20], the stearate route (18 : 0 → 18 : 1 → 18 : 2 → 18 : 3) and the palmitate route (16 : 0 → 16 : 1), the former may operate relatively more actively than the latter. But this was not the case. The activities both of stearoyl-CoA and palmitoyl-CoA desaturases were not considerably reduced to an almost undetectable level in the 24 h-starved cells (Fig. 6), indicating no difference in the relative rates of the two desaturation pathways. An alternative explanation is that it is likely that the elevated level of 2-aminoethylphosphonolipid with much 18 : 3 and the lowered content of phosphatidylethanolamine with much 16 : 1 would largely produce the altered proportions of these two fatty acids. The decrease in phosphatidylethanolamine would result from the loss of intracellular organelles containing this lipid in large amounts due to sequestration by autophagic vacuoles.

On the other hand, it was also observed that following nutritional shift-up (refeeding), a rapid modification of membrane lipid composition occurred. Although there have been many studies dealing with macromolecular events following refeeding of the starved *Tetrahymena* cell [35,37], no information has been available regarding its membrane lipids. Therefore, this is the first report describing the detailed analyses of lipid composition in various membrane fractions during different periods of refeeding. In general, lipid modifications following shift-up were found to occur in a reversed manner as observed during starvation. Within 6 h of refeeding at which there was no active cell division, the overall composition of polar head groups as well as of acyl chains was largely restored to the initial level of control cell membranes prior to starvation. This recovery of the membrane lipid profile was to a large extent accomplished by the increase in phosphatidylethanolamine and 16 : 1 with the compensating decrease in 2-aminoethylphosphonolipid and 18 : 3. The gradual rise in the 16 : 1 content can be accounted for by the enhanced activity of palmitoyl-CoA desaturase in the refed cells. Concurrently, stearoyl-CoA desaturase activity also was regained at a rate comparable to that of palmitoyl-CoA desaturase.

The marked reduction in 18 : 3 appears more complicated to explain. Since the increased 16 : 1 content alone would not be responsible for the relative decrease in 18 : 3, some other mechanisms should be considered for interpreting the starvation-induced decrease of this fatty acid. There are two possibilities. One is that palmitoyl-CoA, the common substrate for reactions of desaturation to form 16 : 1 and chain elongation to form 18 : 0, which is a substrate for the stearate desaturation pathway, would be consumed at a higher rate in the former reaction than in the latter. Thus, despite the rapidly recovering activity of stearoyl-CoA desaturase, the sequential unsaturation processes after the chain elongation no longer proceed. The other possibility is the suppressed activity of two sequences (18 : 1 → 18 : 2 → 18 : 3) followed by the step of stearoyl-CoA desaturase (18 : 0 → 18 : 1), which was indeed reflected

in the substantial increase in the 18 : 1 content and also in the higher radioactivity found in this fatty acid fraction. Our recent studies have demonstrated that a direct desaturation of phospholipid acyl chains occurs in these two steps to synthesize 18 : 2 and 18 : 3 [38]. It could be possible that this desaturation reaction might be selectively inhibited at one or both steps. Further extensive investigations will be needed to determine which mechanism is actually involved in lowering the 18 : 3 content, and are currently in progress.

It is of greater interest to note that such ameliorative lipid modification was coupled with expansion of the surface membrane, pellicle, and with the renewal of some organelles such as mitochondria and endoplasmic reticulum which had been lost during starvation. Indeed, certain ultrastructural findings indicated processes of the membrane growth (see Fig. 1). Shortly after the starved cells with much simplified membrane systems were transferred to nutrient-rich medium, they regained potency of membrane renewal and accomplished within a rather short time ameliorative recovery of both lipid composition and ultrastructure in membranes. Therefore, such a refeeding system would also be a powerful tool to gain knowledge regarding membrane formation. Our earlier experiments with *T. pyriformis* WH-14 cultures showed that the distribution rates of radioactive phospholipids to various target membranes were nearly equal in the three cell systems examined; active growth [39], no growth (stationary phase, metabolically inhibited) [40] and starvation [40]. Such dynamic transfer of lipids between different membrane fractions has been well known with numerous kinds of cell [41]. The results presented in this work have demonstrated that in the refed cells of *T. pyriformis* NT-I there is a more active mobilization of lipids, i.e., exchange, than in the exponentially growing control cells, except that at the early stage the cilia of the refed cells are rather independent of the rapid exchange process, as indicated by the much lower specific activity than that of the control cilia (Fig. 7). This difference can be explained by the finding that during the first 6 h without cell division, there is little or no renewal of cilia in the refed cells, whereas the control cells always produce cilia in accordance with active division. It is reasonable to assume that the rapid transfer of newly made phospholipids should be required for an urgent demand of cells to resume division. As several studies have shown that rapid macromolecular events follow refeeding, there is an early increase in protein synthesis which begins prior to the onset of RNA synthesis [37]. Eventually, one would expect that a rapid and substantial increase of the two principal membrane components, lipids and proteins, contributes both to expansion of the surface membrane, pellicle and to the renewal of intracellular organelles in the refed cell.

Acknowledgements

This work was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, the Japan Society for the Promotion of Science under the Japan-U.S.A. Cooperative Science Research Program, and the Naito Science Foundation, Japan. We are grateful to Y. Kameyama and S. Yoshioka for assistance in assay of desaturases, and also to F. Kato for assistance in lipid analysis.

References

1 Singer, S.J. and Nicolson, G.L. (1972) Science 172, 720—731
2 Sandermann, H. (1978) Biochim. Biophys. Acta 515, 209—237
3 Singer, S.J. (1974) Annu. Rev. Biochem. 43, 805—833
4 Thompson, G.A. and Nozawa, Y. (1977) Biochim. Biophys. Acta 472, 55—92
5 Nozawa, Y. (1980) in Membrane Fluidity: Biophysical Techniques and Cellular Regulation (Kates, M. and Kuksis, A., eds.), pp. 399—418, Humana Press, Inc., Clifton, NJ
6 Kasai, R. and Nozawa, Y. (1980) Biochim. Biophys. Acta 617, 161—166
7 Hamburger, K. and Zeuthen, E. (1957) Exp. Cell Res. 13, 443—449
8 Nozawa, Y. and Thompson, G.A. (1971) J. Cell Biol. 49, 712—721
9 Nozawa, Y. (1975) in Methods in Cell Biology (Prescott, D.M., ed.), Vol. 10, pp. 105—133, Academic Press, New York
10 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
11 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
12 Rouser, G., Siakotos, A.N. and Fleischer, G. (1966) Lipids 1, 85—88
13 Martin, C.E., Hiramitsu, K., Kitajima, Y., Nozawa, Y., Skriver, L. and Thompson, G.A. (1976) Biochemistry 15, 5218—5227
14 Yau, T.M., Buckman, T., Hale, A.H. and Weber, M.J. (1976) Biochemistry 15, 3212—3219
15 Nozawa, Y. and Kasai, R. (1978) Biochim. Biophys. Acta 529, 54—66
16 Fukushima, H., Nagao, S. and Nozawa, Y. (1979) Biochim. Biophys. Acta 572, 178—182
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Nozawa, Y., Fukushima, H. and Iida, H. (1975) Biochim. Biophys. Acta 406, 248—263
19 Koroly, M.J. and Conner, R.L. (1976) J. Biol. Chem. 251, 7588—7592
20 Conner, R.L. and Stewart, B.Y. (1976) J. Protozool. 23, 193—196
21 Nozawa, Y. and Thompson, G.A. (1976) in Biochemistry and Physiology of Protozoa (Levandowsky, M. and Hutner, S.H., eds.), Vol. 2, pp. 276—338, Academic Press, New York
22 Ferguson, K.A., Conner, R.L. and Mallory, F.B. (1971) Arch. Biochem. Biophys. 144, 448—450
23 Ferguson, K.A., Davis, F.M., Conner, R.L., Landrey, J.L. and Mallory, F.B. (1975) J. Biol. Chem. 250, 6998—7005
24 Kasai, R., Sekiya, T., Okano, Y., Nagao, S., Ohki, K., Ohnishi, S. and Nozawa, Y. (1977) Maku (Membrane) 2, 301—312
25 Shimonaka, H., Fukushima, H., Kasai, R., Nagao, S., Okano, Y. and Nozawa, Y. (1978) Experientia 34, 586—587
26 Lees, A.M. and Korn, E.D. (1966) Biochemistry 5, 1475—1481
27 Kasai, R., Kitajima, Y., Martin, C.E., Nozawa, Y., Skriver, L. and Thompson, G.A. (1976) Biochemistry 15, 5228—5233
28 Kitajima, Y. and Thompson, G.A. (1977) Biochim. Biophys. Acta 468, 73—80
29 Fukushima, H., Watanabe, T. and Nozawa, Y. (1976) Biochim. Biophys. Acta 436, 249—259
30 Nandini-Kishore, S.G., Mattox, S.M., Martin, C.E. and Thompson, G.A. (1979) Biochim. Biophys. Acta 551, 315—327
31 Nozawa, Y., Kasai, R., Kameyama, Y. and Ohki, K. (1980) Biochim. Biophys. Acta, in the press
32 Erwin, J. and Bloch, K. (1963) J. Biol. Chem. 238, 1618—1624
33 Wunderlich, F., Speth, V., Batz, W. and Klenig, H. (1973) Biochim. Biophys. Acta 298, 39—49
34 Fukushima, H., Martin, C.E., Iida, H., Kitajima, Y., Thompson, G.A. and Nozawa, Y. (1976) Biochim. Biophys. Acta 431, 165—179
35 Thompson, G.A., Bambery, R.J. and Nozawa, Y. (1972) Biochim. Biophys. Acta 260, 630—638
36 Cameron, I.L. and Jeter, J.R. (1970) J. Protozol 17, 428—431
37 Cameron, I.L., Griffin, E.E. and Rudick, M.J. (1971) Exp. Cell Res. 65, 265—272
38 Watanabe, T., Fukushima, H. and Nozawa, Y. (1979) Biochim. Biophys. Acta 575, 365—374
39 Nozawa, Y. and Thompson, G.A. (1971) J. Cell Biol. 49, 722—731
40 Nozawa, Y. and Thompson, G.A. (1972) Biochim. Biophys. Acta 282, 93—104
41 Wirtz, K.W.A. (1974) Biochim. Biophys. Acta 344, 95—117